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Understanding macrophage differentiation during space flight: The importance of ground-based experiments before space flight

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Abstract

In preparation for a space flight on STS-126, two *in vitro* culture systems were used to investigate macrophage colony stimulating factor-dependent macrophage differentiation from mouse primary bone marrow cells. The patented Techshot Cell Cult Bioreactor and the BioServe Fluid Processing Apparatus (FPA)¹ were operated in different orientations to determine their impact on macrophage growth and differentiation. Bone marrow cell parameters were determined after cells were grown in FPAs incubated at 37°C in vertical or horizontal orientations, and macrophage cell recovery was significantly higher from FPAs that were incubated in the horizontal orientation compared to “vertical” FPAs. Similarly, when bone marrow cells were grown in the Techshot bioreactor, there were significant differences in the numbers of macrophages recovered after 7 days, depending on movement and orientation of the bioreactor. Macrophage recovery was highest when the patented bioreactor was rotated in the horizontal, x-axis plane (merry-go-round fashion) compared

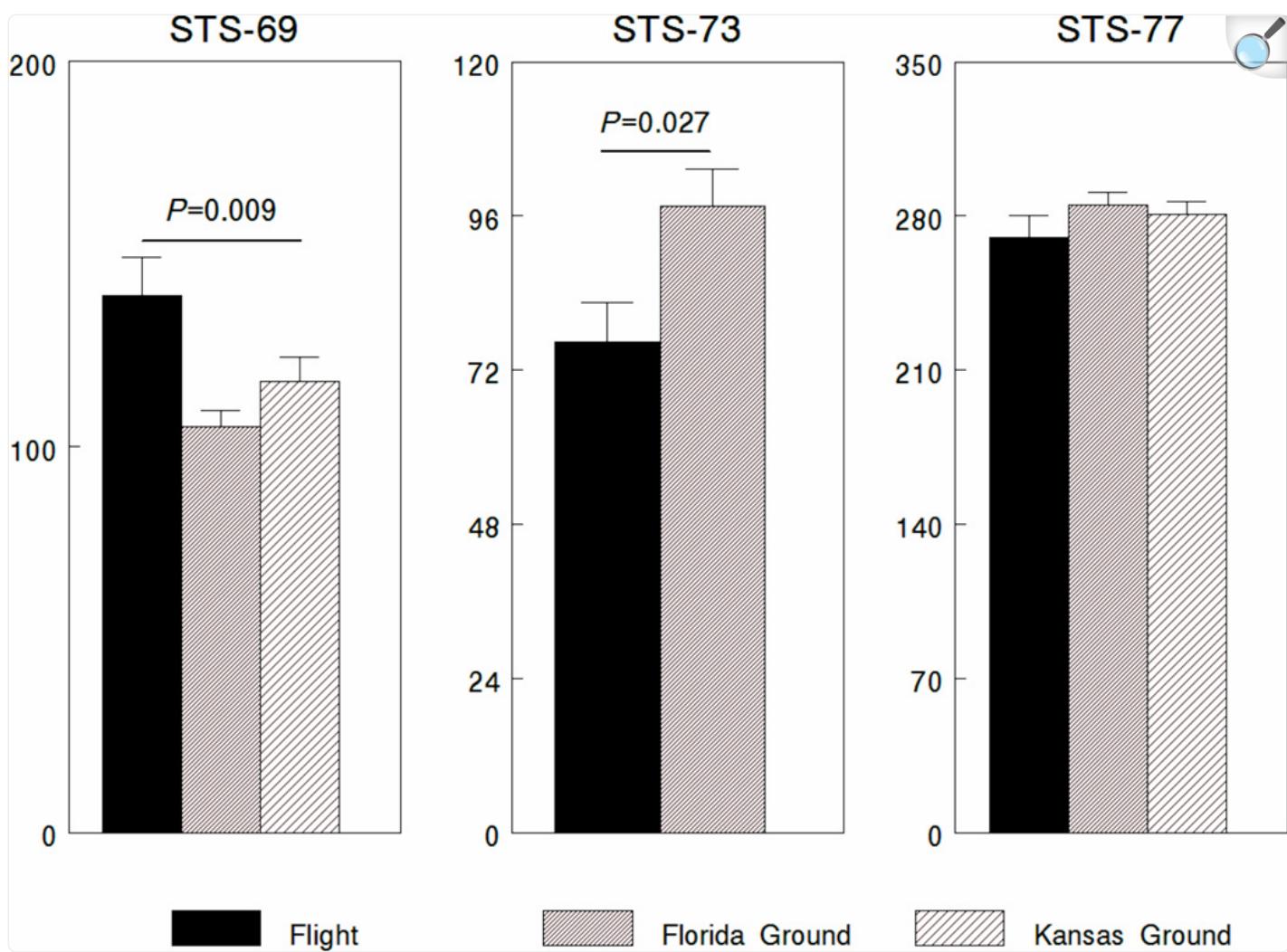
to static and vertically, y-axis plane rotated (Ferris wheel fashion) bioreactors. In addition, the expression of F4/80 and other differentiation markers varied depending on whether macrophages differentiated in FPAs or in bioreactors. After 7 days, significant differences in size, granularity and molecule expression were seen even when the same primary bone marrow cells were used to seed the cultures. These data show that culture outcomes are highly dependent on the culture device and device orientation. Moreover, the impact of the culture system needs to be understood in order to interpret space flight data.

Keywords: cell differentiation, macrophage, fluid processing apparatus, space flight hardware

Introduction

Spaceflight and/or microgravity can alter the immune system at organismic levels [1, 2] as well as cellular levels [3–5]. In particular, the hematopoietic system that generates cells in the monocyte/macrophage lineage may be a target. In 1986, Taylor *et al.* [6] detected monocytopenia post flight in seven out of 11 astronauts that flew on the space shuttle. In addition, some astronauts exhibited changes in insulin-like growth factor-1 receptor expression on monocytes post flight [7]. Changes in colony forming units-granulocyte macrophage (CFU-GM) and colony forming units-macrophage (CFU-M) were seen in rats, post flight on biosatellites [8, 9] and during the Spacelab Life Sciences (SLS)-2 space lab flight on space transportation system (STS)-58. [10]. However, comparison of CFU-M after two 7-day space shuttle flights found that there were inconsistencies in CFU-M in the bone marrow of rats post flight [11]. We also found different outcomes when we measured the development of CFU-M *in vitro* after three different shuttle missions ([Figure 1](#)). During STS-69, a 260 hr. mission, we found that significantly more CFU-M developed in cultures grown in space compared to ground controls. In contrast, during STS-73, a 382 hr space-flight mission, we found the opposite result with inhibited CFU-M generation in space-flight cultures compared to ground controls ([Figure 1](#)). There was no impact of space flight on CFU-M generation during the STS-77 shuttle mission, a 240 hr. mission.

Figure 1. CFU-M development on STS-69, 73 and 77.



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Soft agar colony assays to detect CFU-M (macrophage colony forming units) were conducted in FPAs as described [12]. Colony assays were set up for shuttle flight on STS-69 and 73 (and the associated ground controls) approximately 48 hours prior to turnover to the shuttle loading crew. The FPAs were equilibrated for 12 hours at 37° C and 8% CO₂capped with rubber septa to trap equilibrated air, and shipped at ambient temperature to Kennedy Space Center (KSC) for integration into Group Activation Packs (GAPs) [21]. To control for transportation of the FPAs to KSC, ground controls were conducted at KSC and in our laboratory at Kansas State University (KSU). Colony assays set up for shuttle flight and ground controls for STS-73 were set by harvesting bone marrow cells at KSU which were immediately shipped in RPMI₁₀ (on ice) to KSC. Ammonium Chloride lysis of red blood cells and all subsequent assay procedures were performed in the laboratory facilities at KSC Space Life Science Support Facility (SLSL). These assays were set up approximately 20 hours prior to turnover to the shuttle loading crew. As a result of the assay setup being

conducted at KSC there were no ground controls conducted at KSU.

Space flight experiments are extraordinarily challenging. Not only do you have to modify experimental conditions and equipment for the space-flight environment, the ability to reproduce the exact flight conditions from flight-to-flight is next to impossible. Occasionally, one finds that equipment used for space flight is an advance on the technique used for normal ground experiments [12], but this is rare. Frequently, secondary payloads are at the mercy of the requirements of the primary payloads. Therefore, variables such as temperature, astronaut time for manipulations and time-lines are not optimal.

The assay of macrophage hematopoietic precursors can be done two ways. To identify the number of precursors, bone marrow cells are isolated and cultured in soft agar containing growth factors such as macrophage colony stimulating factor (M-CSF) or granulocyte-macrophage colony stimulating factor (GM-CSF) at appropriate concentrations [13]. Assessment of how many colonies grow provides an estimate about the number of CFU-M or CFU-GM that are present in the stem cell population [14]. Alternatively, macrophages can be induced to differentiate in M-CSF and the cells that differentiate into macrophages in 6–8 days can be phenotyped based on their cell-surface molecules, assayed for functional assays, or used for other kinds of analyses [15].

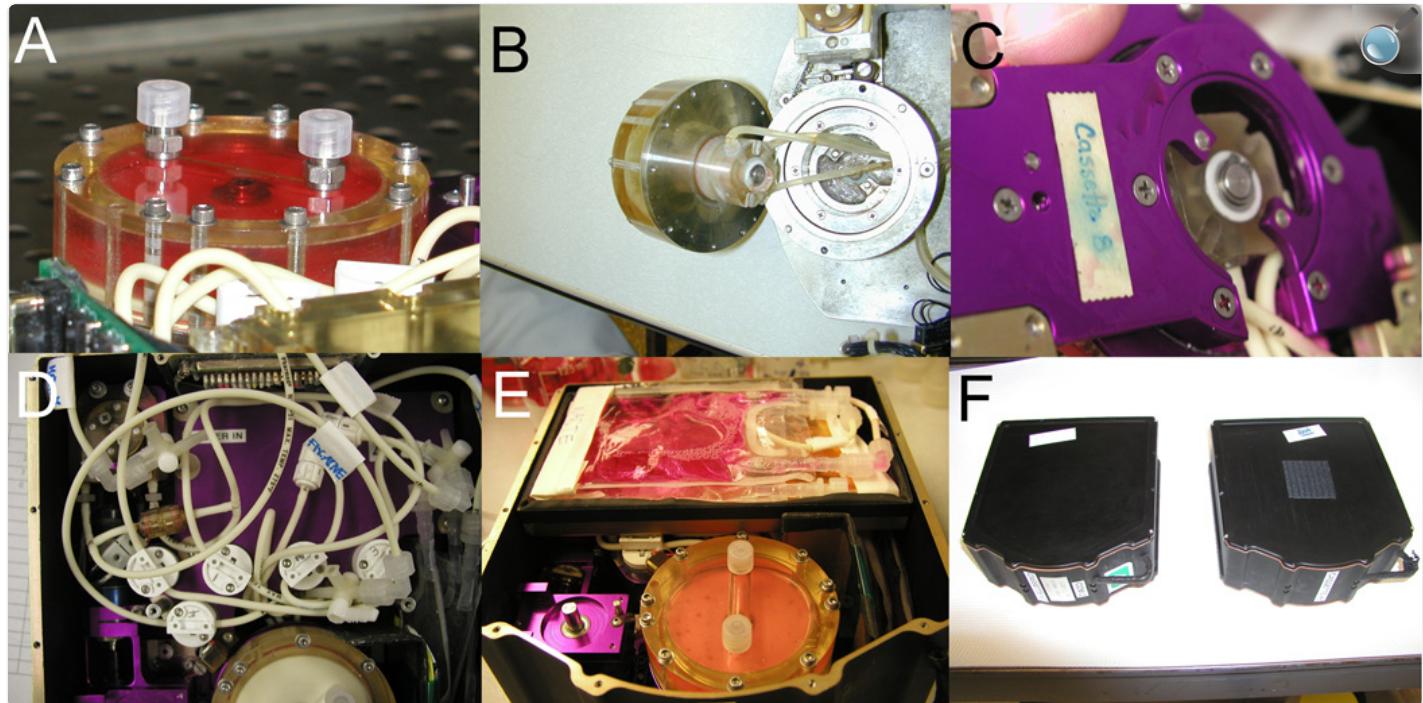
Our group had an opportunity to determine if macrophage development was affected *in vitro* on the space shuttle flights STS-57, STS-60 and STS-62 [16]. Mouse bone marrow stem cells were incubated in liquid culture in medium containing M-CSF during the space flights. The differentiated macrophages were fixed during flight and phenotyped by flow cytometry and the supernatants were collected from viable cells and were assayed for interleukin-6 (IL-6) post flight. However, because these experiments were secondary payloads, the temperature conditions were not physiological and some questions about the results remained[16]. Therefore, we explored additional opportunities to attempt to resolve the issue about the impacts of space flight on macrophage development at the cellular level.

Preflight Verification Tests

Although space flight offers the opportunity to do research in microgravity, there is also the challenge of adapting tissue culture methods for the space environment. For our experiments, two possible hardware configurations were tested. One was the Cell Cult Bioreactor from Techshot (Formerly Space Hardware Optimization Technology, Inc. Greenville, IN) [17–19]. The patented bioreactor vessel [19] holds approximately 50 ml of culture medium and is approximately 6 cm (diameter) × 2.7 cm (height) in size (Figure 2). The bioreactor in its final assembled configuration was computer controlled with flexible feeding and collection schedules and volumes. The bioreactor could be rotated at 1 revolution per minute (RPM) during flight to allow for adequate mixing of fresh culture medium or fixative. We also tested the BioServe Fluid Processing Apparatus (FPA)[16, 20–22]. These culture vessels are 1.35 cm diameter (1.31 mm

thickness) glass barrels that are 11.70 cm long ([Figure 3 A-C](#)). Transfer of the medium from one chamber to another is accomplished through a bypass on one side of the tube ([Figure 3A](#)). These culture devices allow the incubation of cells in a volume of 3.0 ml which can be supplemented with one or two additional 1.5 ml medium supplements or one feeding and the addition of fixatives/preservatives. Detailed set up of the equipment for bone marrow cells has been described by our group previously [[5](#), [16](#)].

Figure 2. Bioreactor configuration.



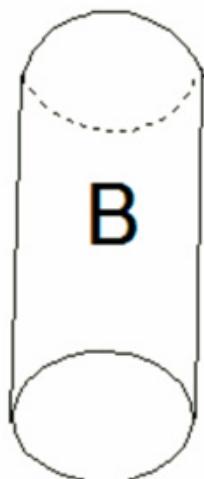
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The Techshot bioreactor vessel is a 6 cm (diameter) × 2.7 cm (height) culture vessel (Panel A) that is connected via rotary unions to medium feeding lines (Panel B, removed and viewed from below). The bioreactor is served by peristaltic pumps (Panel C) and computer controlled solenoid valves (Panel D) that control fresh medium or waste flow from or into Teflon bags (Panel E). The completed assembly (Panel F) can be inserted into a middeck-sized locker for use in space flight [[17](#), [18](#)].

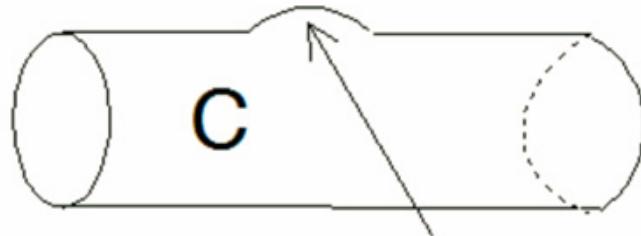
Figure 3. Fluid Processing Apparatus and Techshot bioreactor orientations.

FPA

A

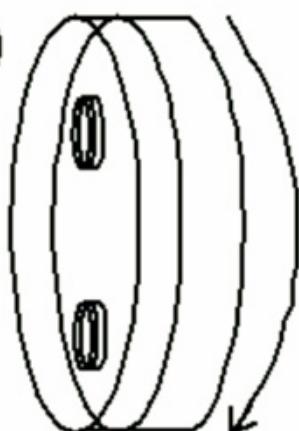


Vertical Orientation



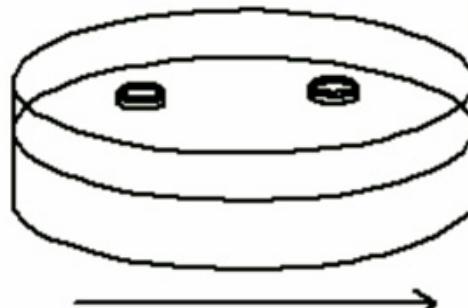
bypass
Horizontal Orientation

D



Ferris Wheel
y-axis

E



Merry-go-round
x-axis

Techshot bioreactor

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FPA diagram (A) shows a loaded example in which 1×10^7 cells were placed in three ml of medium in chamber 1 (yellow) separated by two rubber septa. The FPA is engineered with a bypass so that when the second septum from the right crosses the bypass during motion from left to right the medium in the second chamber (red, 1.5 ml) will mix with the material in chamber 1 and the first two septa from the right will compress together. The next two septa also compress with additional movement to mix the contents of chamber 3 (green, 1.5 ml) with the contents previously mixed; showing vertical (B) or horizontal (C) orientation. The Techshot bioreactor was incubated in a vertical (Ferris wheel) or (E) horizontal (merry-go-round) configurations and rotated at 1 RPM.

There were significant differences in the complexity and capabilities of the culture systems available to us which presented some questions about the data each system was capable of providing. This is not a new concept. Others have compared hardware in ground based experiments to determine strengths and weaknesses of systems like rapidly rotating clinostats and random positioning machines [23]. For shuttle-flight experiments, most comparisons are made to samples incubated on Earth, although some space-flight experiments have been developed with controls in 1xg centrifuges that were also in space [24–26]. Comparisons have also been made with animals subjected to space flight and antiorthostatic suspension on Earth [9]. However, for our *in vitro* experiments, the orientation of the culture devices became a concern to us, especially for our Earth controls. In particular, the bioreactor could be oriented in “Ferris wheel” ([Figure 3D](#)) or “merry-go-round”([Figure 3E](#)) orientations. We compared cell recovery { % cell recovery = (# viable cells recovered/ # viable cells added to the culture vessel)x100} from bioreactors that were rotated (at 1 RPM) or kept stationary that were incubated in both horizontal and vertical orientations ([Table 1](#)). One-third or fewer viable cells were recovered from bioreactors that were not rotated compared to bioreactors that were rotated at 1 RPM. In addition, approximately 80% more viable cells were obtained from bioreactors incubated in the horizontal orientation compared to the vertical orientation ([Table 1](#)). When we differentiated macrophages in FPAs incubated in vertical ([Figure 3B](#)) or horizontal orientations ([Figure 3C](#)), we also found that there were differences in the numbers of cells recovered. We averaged $3.7 \pm 0.9 \times 10^6$ cells per FPA after 7 days in M-CSF (15 ng/ml) when FPAs were stored in the vertical position. In contrast, we averaged $1.2 \pm 0.3 \times 10^7$ cells per FPA when the FPAs were stored in a horizontal position when bone marrow cells were differentiated into macrophages in medium containing M-CSF. The cell input recovered was 120% vs. 37% for horizontal and vertical FPAs, respectively. In general, the surface area that cells had to spread on was greater in both the bioreactor and the FPAs when the culture units were in the horizontal compared to the vertical orientation. It suggests that cell recovery is improved with greater surface area, independent of fluid volume. FPA’s were generally more efficient in viable cell numbers recovered compared to the larger bioreactors.

Table 1.

Recovery of cells from bioreactor

	Horizontal ¹		Vertical ¹	
	Rotation	Stationary	Rotation	Stationary
Viable Cells Recovered ²	$1.6 \pm 0.3 \times 10^7$	$3.6 \pm 0.5 \times 10^6$	$3.2 \pm 0.5 \times 10^6$	$1.1 \pm 0 \times 10^6$
% Cell input Recovered ³	5.3%	1.2%	1.1%	0.4%

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¹ 3×10^8 primary mouse bone marrow cells were seeded in a chamber volume of 50 ml containing 15ng/ml mCSF.

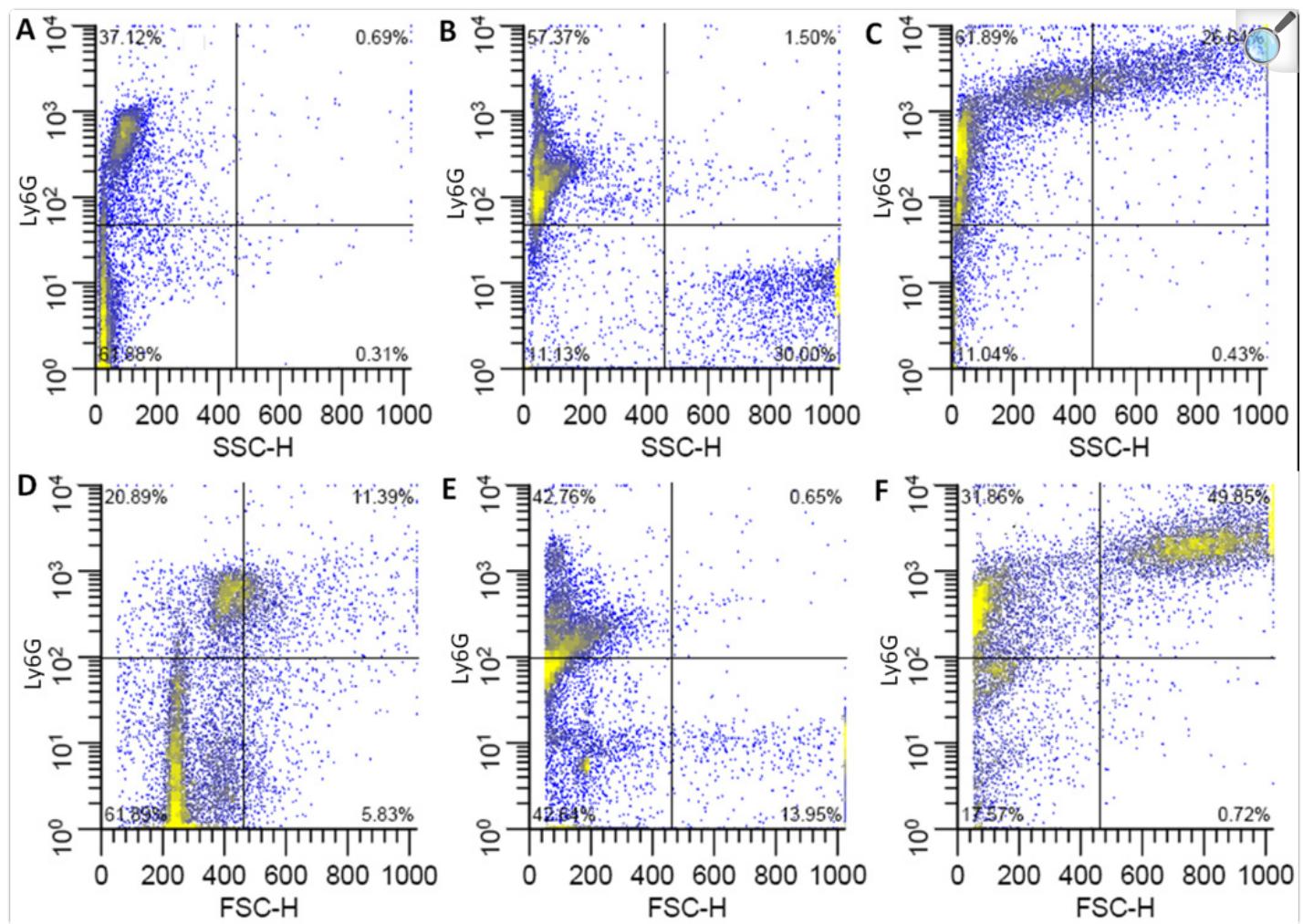
²Cells were incubated for 7 days. They were fed with 15 ml fresh medium on days 3 and 5.

³% Cell input Recovered=(# of viable cells recovered/# viable cells added to the culture vessel) $\times 100$

We examined the cell surface phenotype of macrophages that differentiated in FPAs or the bioreactor. Cells were incubated in 15 ng/ml of M-CSF for 7 days and assessed for the expression of several cell surface molecules commonly found on macrophages. Both the FPAs and bioreactors were oriented in the horizontal positions and the bioreactors were rotated at 1 RPM. The bioreactors were fed with 15 ml of medium (with M-CSF) on days 3 and 5. The FPAs were stationary except when they were fed with 1.5 ml of medium (with M-CSF) on days 3 and 5 of the 7 day culture period. Cells were stained with fluorochrome-labelled antibodies specific for macrophage surface proteins c-fms [27–29], Mac-2 [30, 31], F4/80 [32, 33] and Ly6G [34–36]. Freshly harvested bone marrow cells are generally smaller with several subpopulations of cells, reflecting the large number of neutrophils in mouse bone marrow [37] and other populations of cells including undifferentiated stem cells [38–41]. In FPAs the cells expressing Ly6G fall into two subpopulations. There is a small, less granular subpopulation and a large, granular Ly6G-negative subpopulation ([Figure 4B and E](#)). This contrasts bone marrow macrophages that differentiate in bioreactors which were mostly Ly6G positive and had continuums of sizes and granularities ([Figure 4C and F](#)). For F4/80 expression, most of the cells recovered from FPAs and bioreactors were F4/80 positive ([Figure 5](#)). However, the cells in FPAs were smaller and agranular ([Figure 5B and E](#)) compared to cells collected from bioreactors after 7 days of differentiation, which expressed a continuum of granularities and sizes ([Figure 5C and F](#)). We saw similar trends for Mac-2 ([Figure 6](#)) and c-fms ([Figure 7](#)) expression. Macrophages were clearly differentiating in both culture devices over the 7-day differentiation period. However, the data clearly demonstrate that the properties of the cells were very distinct based on size, granularity and antibody

staining. This is an important insight because it illustrates that just like the number of macrophages that develop in each type of culture device, the cell phenotype is hardware dependent. It is not clear why the cell phenotypes were so distinct when bone marrow cells were incubated in the two different hardware. The smaller fluid volume of the FPA may have had an impact. Although the numbers of cells in each of the hardware (1×10^7 vs. 3×10^8) was adjusted, the final cell concentration in an FPA was 1.7×10^6 cells/ml (after all septa movements) compared to 6×10^6 cells per ml in the Techshot bioreactor. Perhaps the increased cell concentration induced more culture stress. Alternatively, the distinct shapes of the devices may have influenced the data. This hypothesis is supported by the experiments presented here. When we changed the orientations of FPAs or the Techshot bioreactor, there were distinct changes in the culture outcomes. Additional experiments will be needed to test these hypotheses.

Figure 4. Flow cytometric analysis of Ly6G on bone marrow derived macrophages.

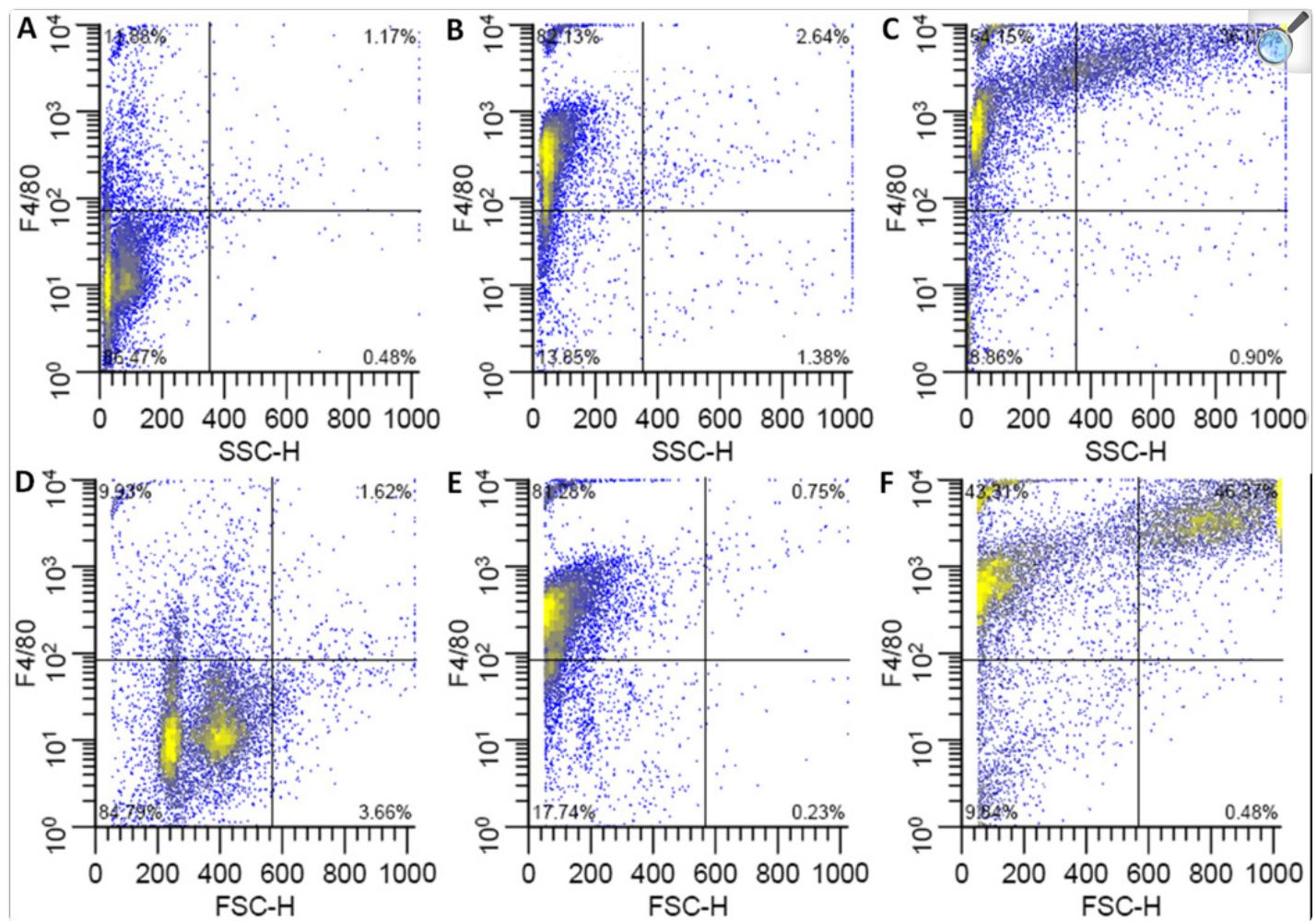


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Representative plots of Ly6G fluorescence vs. side scatter (top row) or forward scatter (bottom row).

Undifferentiated, freshly harvested bone marrow cells (A, D), or cells differentiated in medium containing 15 ng/ml M-CSF in FPAs (B, E) or Bioreactors (C,F). Culture vessels were incubated in horizontal orientations.

Figure 5. Flow cytometric analysis of F4/80 on bone marrow derived macrophages.

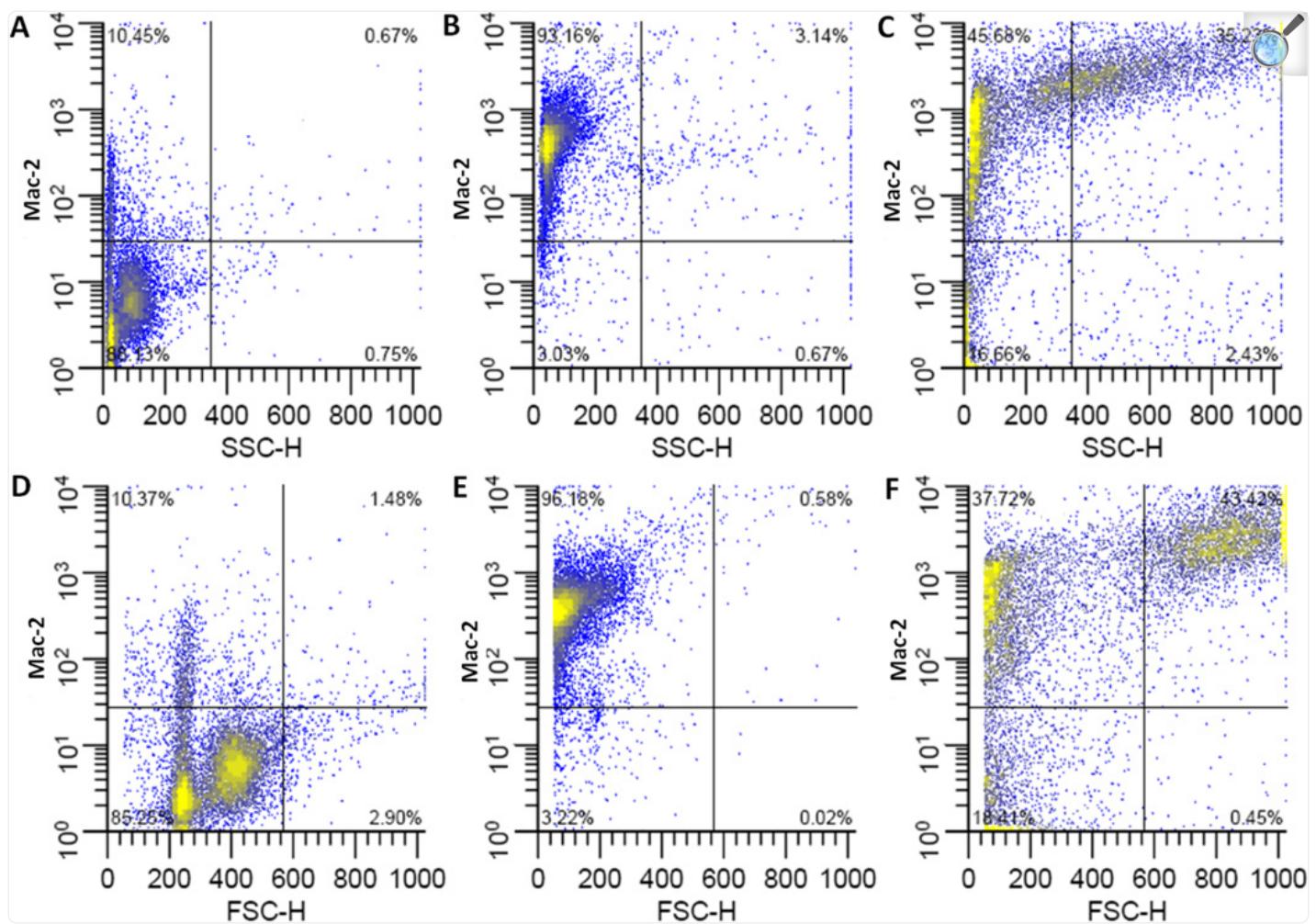


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Representative plots of F4/80 fluorescence vs. side scatter (top row) or forward scatter (bottom row).

Undifferentiated, freshly harvested bone marrow cells (A, D), or cells differentiated in medium containing 15 ng/ml M-CSF in FPAs (B, E) or Bioreactors (C, F). Culture vessels were incubated in horizontal orientations.

Figure 6. Flow cytometric analysis of Mac-2 on bone marrow derived macrophages.

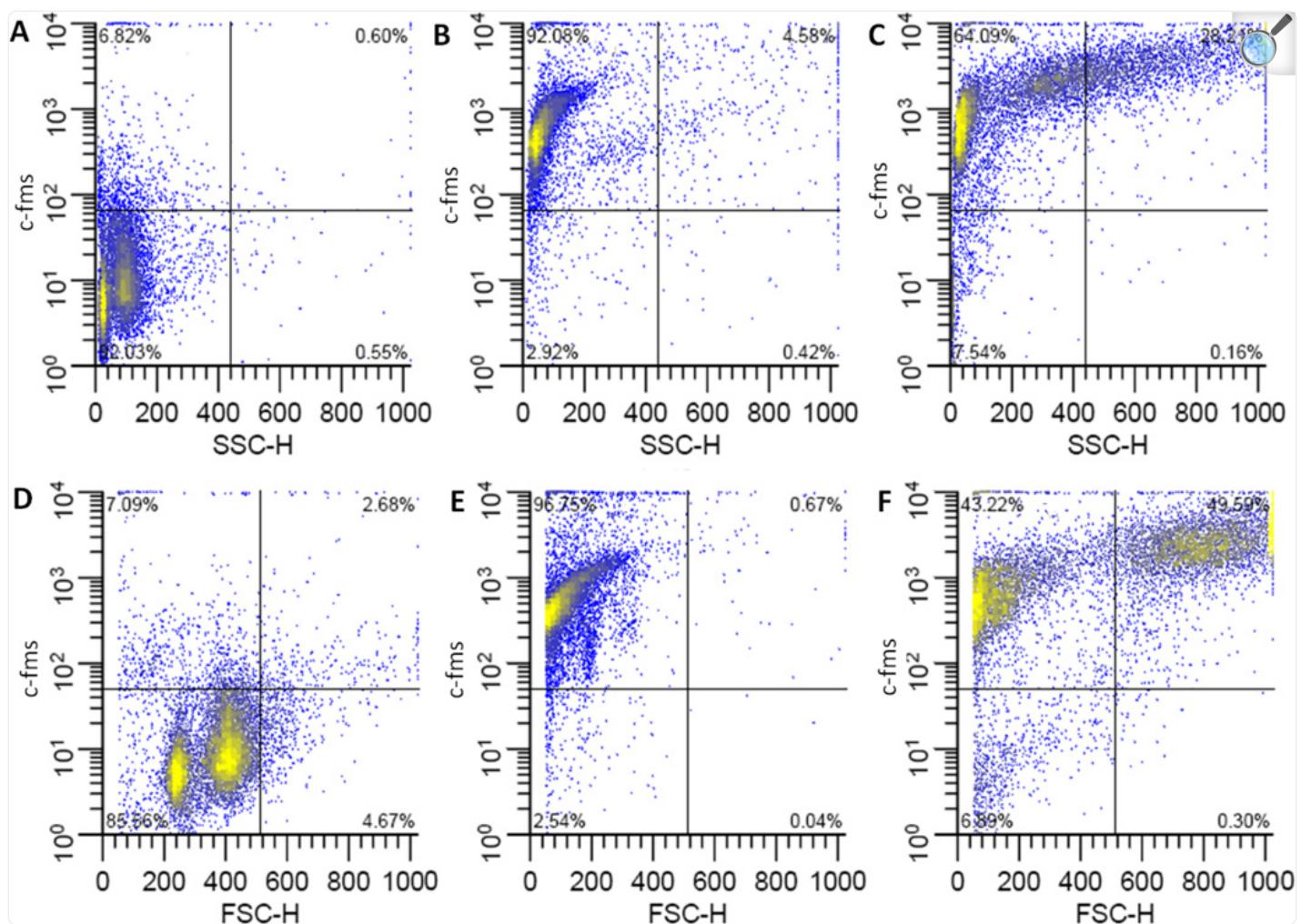


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Representative plots of Mac-2 fluorescence vs. side scatter (top row) or forward scatter (bottom row).

Undifferentiated, freshly harvested bone marrow cells (A, D), or cells differentiated in medium containing 15 ng/ml M-CSF in FPAs (B, E) or Bioreactors (C,F). Culture vessels were incubated in horizontal orientations.

Figure 7. Flow cytometric analysis of c-fms on bone marrow derived macrophages.



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Representative plots of c-fms fluorescence vs. side scatter (top row) or forward scatter (bottom row).

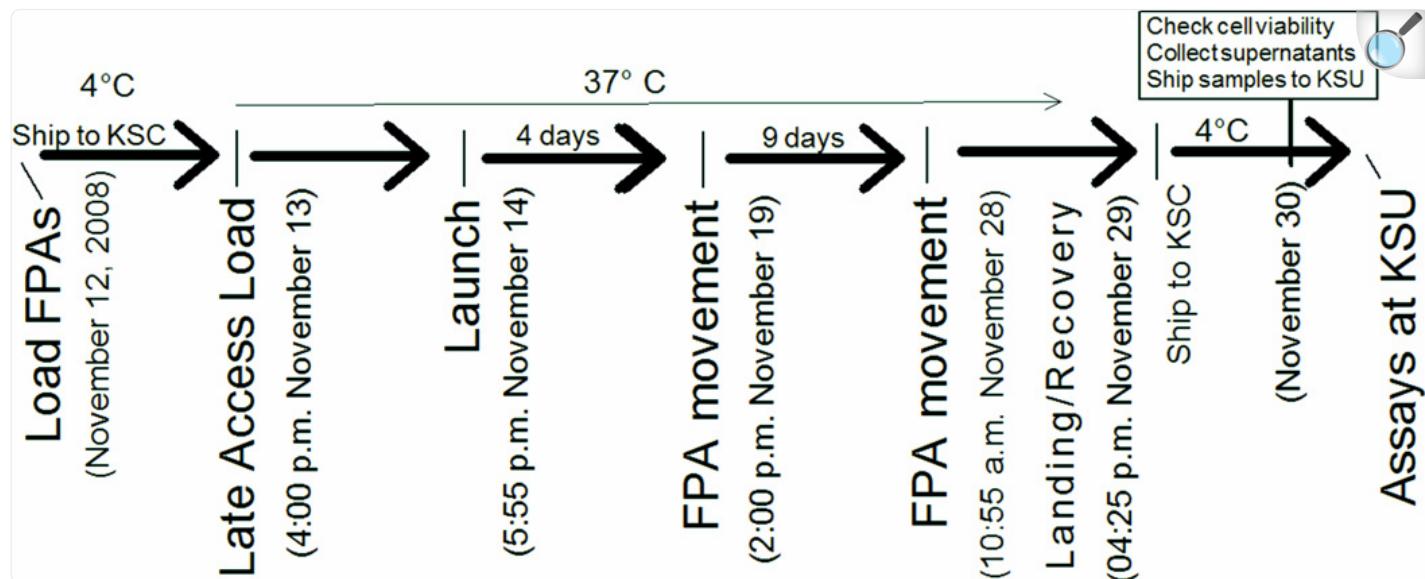
Undifferentiated, freshly harvested bone marrow cells (A, D), or cells differentiated in medium containing 15 ng/ml M-CSF in FPAs (B, E) or Bioreactors (C, F). Culture vessels were incubated in horizontal orientations.

STS-126

We initially developed our experiment for the Techshot bioreactor. However, the loss of the space shuttle Columbia on February 1, 2003 and the announcement on April 4, 2006 of a change in the NASA strategic plan [42], restricted access to the space shuttle laboratory. In 2008, we secured another opportunity to fly an experiment on the space shuttle Endeavour, STS-126. This 14-day flight provided the opportunity to assess bone marrow macrophage differentiation *in*

vitro in response to recombinant mouse M-CSF using FPA hardware[5]. Cells were fixed with formalin and RNA was preserved at day 14 of the space flight (Figure 8). Cells were returned to Earth for cell surface molecule assessments and transcriptional arrays were performed using the Affymetrix Mouse Genome 430 2.0 array [5].

Figure 8. Time-line of activities for bone marrow macrophage differentiation on STS-126.



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The FPAs were prepared as previously described [5] and were shipped to the NASA SLSL at KSC 2 days before launch. The FPAs were loaded into GAPs and placed at 37°C in the Commercial Generic Bioprocessing Apparatus (CGBA). Parallel temperature and activation profile conditions were maintained on GAPs kept at the SLSL. The cells in the FPAs were re-fed with 1.5 ml of medium supplemented with rmM-CSF on day 5 of the spaceflight by combining chambers 1 and 2 (See Figure 3). On day 14 of spaceflight, the content of the FPAs' third chamber were mixed with the cell suspension in the previously merged chambers to preserve RNA or to fix or maintain cells until processing after landing. STS-126 landing occurred at the Edwards Air Force Base in California after 16 days of space flight. Samples were placed at 4°C and transported to the SLSL in Florida.

We discovered that cells proliferated more in space compared to ground-incubated cells. There were also several changes in cell surface molecules on macrophages differentiated in space compared to ground controls [5]. We saw significant shifts in the distribution of cells expressing CD31, Ly6C, CD11b, F4/80, Mac-2 and c-fos in cells

differentiated in space flight compared to ground controls [5].

The transcript levels in macrophages differentiated in space were also different from those inground controls. Six hundred and seven genes had transcript levels >1.5 fold higher for flight samples than ground controls. In contrast, we found that 1071 genes had gene transcript levels >1.5 fold lower than ground controls ($P < 0.05$)[5]. The genes were sorted into biological function categories using Ingenuity Pathway Analysis (IPA) software and 33 genes were identified in the M-CSF/c-fms pathway. Bone marrow stem cells differentiate into macrophages in the presence of M-CSF in an ordered fashion from myeloid pluripotent precursors to monoblasts to macrophages (Figure 9). Gene expression occurs concomitantly during this process [43–46]. When we compared transcript levels of genes that were higher or lower in cells differentiated during space flight compared to ground controls, we found several genes that were significantly up or down regulated in the M-CSF/c-fms pathway (Figure 9). Moreover, the down regulation of *Rbl-1* and *E2f4* transcripts would be expected because of their roles in proliferation [47, 48] and cell differentiation [49]. *Hoxb3* and *Hoxb4* molecules are necessary for myeloid cell proliferation early in differentiation [44, 45] and the patterns of the transcripts seen in the space-flight samples were consistent with the increased proliferation we detected in space flight compared to ground controls [5].

Figure 9. Transcriptional profile of bone marrow cells as they differentiate into macrophages.

	Commitment				Early differentiation			Late differentiation or Maturation	
	Stem cell	Myeloid pluripotent precursor (GEMM-CFU)	Monocyte-Granulocyte precursor (GM-CFU)	Monocyte precursor (M-CFU)	Monoblast	Pro-monocyte	Monocyte	Immature Macrophage	Macrophage
					c-Fms, Ly6C, CD31	c-Fms, Ly6C, CD31	c-Fms, Ly6C, CD11b, CD31, F4/80	c-Fms, Ly6C, CD11b, Mac2, CD31, F4/80	c-Fms, CD11b, Mac2, CD31, F4/80
Transcript levels [Change from ground control]		<i>Gata1</i> [↓] <i>Gata2</i> [↓] * <i>Hoxb3</i> [↑]* <i>Hoxb4</i> [↑]* <i>Scly</i> <i>Myc</i> <i>Zbtb16</i>	<i>Csf1r</i> <i>Scly</i> <i>Cebpa</i> <i>Myb</i> <i>Myc</i> <i>Runx1</i>	<i>Csf1r</i> <i>Sfpi1</i> <i>Egr1</i> <i>Ets1</i> <i>Ets2</i> <i>Hoxb7</i> <i>Cebpa</i> [↓] <i>Jun</i> <i>Fos</i> <i>Myb</i> [↓] <i>Myc</i>	<i>Csf1r</i> <i>Sfpi1</i> <i>Egr1</i> <i>Ets1</i> <i>Ets2</i> <i>Myc</i> [↓] <i>Stat3</i>	<i>Csf1r</i> <i>Sfpi1</i> <i>Egr1</i> <i>Hoxb7</i> <i>Tnf</i> <i>Ets1</i> <i>Ets2</i> <i>Cebpb</i> <i>Jun</i> <i>Fos</i> <i>Runx1</i>	<i>Csf1r</i> <i>Sfpi1</i> <i>Egr1</i> <i>Hoxb7</i> <i>Cebpb</i> <i>Tnf</i> <i>Ets1</i> <i>Ets2</i> <i>Stat3</i> <i>Jun</i> <i>Fos</i> <i>Runx1</i> <i>Irf1</i>	<i>Csf1r</i> <i>Sfpi1</i> <i>Tnf</i> <i>Egr1</i> <i>Cebpb</i> <i>Hoxb7</i> [↑] <i>Fos</i> [↓] <i>Ets1</i> [↑] <i>Ets2</i> [↑] <i>Stat3</i> [↓] <i>Rbl1</i> [↓]* <i>Irf1</i> <i>E2f4</i> [↓]* <i>Runx1</i> <i>Jun</i> [↑]	<i>Csf1r</i> [↓] * <i>Sfpi1</i> [↑] <i>Egr1</i> [↑]* <i>Cebpb</i> [↓] <i>Tnf</i> [↓] <i>Chrac1</i> [↓] <i>Runx1</i> [↓]* <i>Irf1</i> [↓]
Days in M-CSF	0	1	2	3	4	4.5	5	6	≥7 [F to G] ¹

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Correlation of macrophage differentiation stage (top 3 rows) with known transcriptional activation during differentiation (4th and 5th row).¹ Arrows [↑ or ↓] indicate transcript levels of macrophages differentiated for 14 days in space during STS-126 compared to ground controls. ↑ or ↓ trends are noted only at the last known stage of transcriptional activity during differentiation [43]. * indicates significantly different from ground controls (≥ 1.5 fold) after Benjamini-Hochberg correction [51], $P < 0.05$.

Current and Future Developments

The space flight experiment on STS-126 was a last-minute opportunity. The preflight verification tests comparing the Techshot bioreactor to FPAs prepared us for the flight. In our proposal to NASA, we showed that we could obtain meaningful data using the FPA hardware and that we understood the culture system. Results from STS-126 were validated by the consistency between the transcript expression data and the functional cell proliferation data [5]. This also provides strong justification for NASA to provide investigators ample opportunities to validate their culture systems because the acquired data may be dependent on the culture system. This will be especially important because of

the retirement of the space shuttle and the readjustment of cell culture systems to new space platforms [50].

Acknowledgements

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Footnotes

¹**List of abbreviations.** Colony forming units-granulocyte macrophage (CFU-GM); Commercial Generic Bioprocessing Apparatus (CGBA); Fluid Processing Apparatus (FPA); Granulocyte-macrophage colony stimulating factor (GM-CSF); Group Activation Packs (GAPs); Ingenuity Pathway Analysis (IPA); Interleukin-6 (IL-6); Kansas State University (KSU); Kennedy Space Center (KSC); Macrophage colony forming units (CFU-M); Macrophage colony stimulating factor (M-CSF); Revolution per minute (RPM); Space Life Science Support Facility (SLSL); Space transportation system (STS).

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